Isolation of bacteria from compost for potential use in biodecaffeination

Honors Thesis

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Abstract

Caffeine (1,3,7-trimethyl xanthine) is a bioactive compound present in common foods and beverages such as coffee, tea, and chocolate. Though many consumers value caffeine for its physiological effects, others may prefer decaffeinated products to avoid caffeine. Current methods used to remove caffeine from foods have several disadvantages, such as cost, waste, and solvent use. Enzymatic methods, known as *biodecaffeination*, have been explored as alternatives. Various microbes that are able to metabolize caffeine have been isolated from coffee plant roots. To this point, compost has not been looked at as a potential source for decaffeinating microbes.

Our objective was to isolate and identify microorganisms from compost and to address their potential as biodecaffeinators. Compost samples were obtained and then grown in nutrient broth. Cultures were streaked onto a selective agar that contained caffeine as its only carbon source. Partial 16S rDNA sequences were obtained for a subset of the colonies isolated from the caffeine agar. Preliminary results suggested that the isolated strains were *Pseudomonas*, *Arthrobacter*, *Sphingobacterium*, *Streptomyces*, and *Paenibacillus*, and that they may be capable of metabolizing caffeine.

Key Words: Biodecaffeination, caffeine, 16s rDNA, *Paenibacillus*, *Streptomyces*

Introduction

The removal of caffeine (1,3,7-trimethyl xanthine) from foods where it naturally occurs, such as coffee, tea, chocolate, and cola plants, is of great economic and environmental interest (Babu 2007). Decaffeinated products are becoming increasingly popular, with the market growing by approximately 3% annually. Decaffeinated coffee and tea currently represent 10-15% of their respective global markets (Babu and others 2012a).

Traditional solvent-based decaffeination methods date back as early as 1903 and work by dissolving caffeine from the food using one of several common solvents (Babu 2007). This method is effective at removing caffeine but is unfriendly to the environment, due to the use of toxic solvents in some cases; costly, if the solvent cannot be reused; and unspecific, meaning that other important molecules may also be removed (Babu 2007). Four solvents are primarily used to extract caffeine: dichloromethane, ethyl acetate, supercritical carbon dioxide, and water (as part of the Swiss water method). Dichloromethane and ethyl acetate are able to effectively dissolve the caffeine, though these may remove other important flavor compounds (Babu and others 2012a). While the toxic solvents are almost entirely removed from the decaffeinated product, their usage has recently decreased due to potential health concerns (Babu 2007). Although the water method avoids the issue of using potentially toxic chemicals, it retains the disadvantage of removing other flavors. Similarly, the use of supercritical carbon dioxide has been shown to reduce the concentration of flavorful volatiles in green tea, though it too avoids the use of toxic solvents (Lee and others 2007). The development of new decaffeination processes that are inexpensive and non-toxic may have environmental benefits, as coffee

producers could more easily recycle their caffeine-rich yet otherwise high-carbohydrate and high-protein waste without harming the ecosystem (Gummadi and others 2012).

Caffeine metabolism

Caffeine metabolism has been known to occur in humans and other animals based on the analysis of caffeine by-products excreted in urine (Babu 2007). Cytochrome P450 enzymes are responsible for caffeine breakdown in humans, producing mostly 1-7-dimethylxanthine (84%) and theobromine (12%). Similarly, it is thought that cytochrome P450 is responsible for the catabolism of caffeine in fungi and yeast (Babu 2007; Sauer 1982). Caffeine-producing plants have also been known to contain mechanisms to remove excess caffeine build-up in their tissue. However, the enzymes that are utilized by plants to degrade caffeine have yet to be successfully harvested or purified without destabilizing them, making other enzymatic routes (e.g. bacterial sources for enzymatic decaffeination) more promising.

Penicillium roqueforti was among the first microorganisms to have a reported caffeine metabolizing activity, which was first discovered in 1971 (Schwimmer and others 1971). Researchers grew *P. roqueforti* from the air on agar containing salts, sucrose, and 0.01 M caffeine. After initial growth, cultures were transferred to medium inoculated with a variety of purines. *P. roqueforti* was able to degrade over half of the initial concentration of labeled caffeine in 6 h. Metabolism occurred between concentrations of 0.001 M and 0.04 M caffeine with a maximum growth at 0.008 M. Since its initial discovery, *Penicillium* species have been shown to degrade caffeine with almost 100% efficiency, given optimal conditions at 25ºC (Babu 2007). Other fungi that

have been found to degrade caffeine include *Aspergillus* sp. (*A. niger, A. tamari*, *A. fumigatus*), *Penicillium commune*, *Phanerochaete*, *Rhizopus*, and *Stemphyllium*. It is generally hypothesized that the decaffeination activity of fungi is due to a cytochrome P450 enzyme. However, the large cofactor requirement and relatively slower caffeine degradation rates of cytochrome P450 enzymes make them less viable than bacterial enzymes. Also, the intake of caffeine has been shown to be prevented by urea and ammonium sulfate in *Aspergillus niger* and *Penicillium* spp. (Babu 2007).

Pseudomonas species have been shown to use caffeine as a source of carbon and nitrogen for anabolic processes as early as 1975 (Woolfolk 1975). These studies looked specifically at *Pseudomonas putida* by growing various stock cultures on caffeine-containing media. Carbon in the agar and ammonium added to the media must not have been assumed to be significant or available sources of carbon or nitrogen, respectively. The ability of certain microorganisms to degrade agar, however, challenges this assumption (Hosoda and others 2003). Caffeine metabolism was confirmed by tracking the concentration of labeled [1-methyl-^{14}C]caffeine. It was not reported if other species were tested or if any other species were found unable to grow on caffeine media. *Pseudomonas fluorescens* was also an early target of this research and was among the first *Pseudomonas* species to have a caffeine metabolism confirmed (Babu 2007). A pathway was eventually developed for caffeine metabolism based on some of these initial results (Yamaoka-Yano and Mazzafera 1999; Gummadi and others 2012; Figure 1).

Baker and others (2012) investigated the decaffeinating activity in *Pseudomonas* species and attempted to optimize some physical parameters for biodecaffeination. Microorganisms were isolated from the stems, roots, and leaves of *Coffee arabica* L

plants. Both Gram-positive and Gram-negative species were isolated from the plant but only Gram-negative species exhibited any caffeine-metabolizing ability. The isolates were all found to be members of the *Pseudomonas* family based on biochemical tests. Samples were then grown in caffeine-containing broth, and the residual caffeine concentration was quantified over time using high-performance liquid chromatography.

The effects of pH, temperature, and rotation per minute (rpm) were studied. Optimal pH was found to range from 8.0 to 9.5, at which the authors noted a maximum rate of decaffeination. No specific optimal temperature was given. Caffeine metabolism was most efficient at 180 rpm. With these conditions, the authors reported a 98.6% efficient decaffeination of caffeine liquid medium in an unspecified time frame. They concluded that this high rate of decaffeination represented a new maximum and that this was promising for potential industrial food and beverage applications.

Further work was subsequently done to try to characterize the enzymes responsible for caffeine metabolism in bacteria. The caffeine demethylase enzymes have been difficult to isolate due to their heat lability, and therefore the three-dimensional structure of these enzymes has only been predicted in the scientific literature based on sequence. For example, Babu and others (2012b) were able to deduce the sequence of a caffeine demethylase purified from *Pseudomonas alcaligenes* and found that it had a Fe-S center, which is characteristic of enzymes that bind oxygen for NADPH oxidation. However, the structure they proposed could only be predicted due to its instability at benchtop conditions.

Caffeine dehydrogenase enzymes found in *Pseudomonas* sp. strain CBB1 were also studied and could offer an alternate route for decaffeination. Yu and others (2008)

isolated a *Pseudomonas* sp. strain CBB1 that was able to utilize caffeine as its sole available carbon and nitrogen source. Caffeine dehydrogenase had an optimal activity at a pH of 7.0 and actually increased in effectiveness up to 60ºC, the maximum temperature tested. It was found that this enzyme was more specific to caffeine than it was to other xanthines, and that it could oxidize caffeine into trimethyluric acid. Though they were able to purify the enzyme for use in experiments, the caffeine dehydrogenase would subsequently lose its activity after only a few days at -80ºC storage. Caffeine dehydrogenase's initial heat stability at 60ºC suggests some potential industrial application, though its denaturation after low temperature storage may prove insurmountable for these technologies.

Though caffeine degradation in *Pseudomonas* species typically occurs by a demethylation of caffeine at the C-1, C-3, or C-7 position, other microorganisms have been shown to break down caffeine via alternate mechanisms. For example, *Serratia marcescens* was found to follow the initial demethylation mechanisms but would vary in its caffeine metabolism at later stages, such as in the production of a methyl uric acid intermediate (Mazzafera and others 1996). Madyastha and Sridhar (1998) showed that both *Klebsiella* and *Rhodococcus* species were able to oxidize caffeine at the C-8 position on the purine ring. This leads to cleavages of the two rings, creating an open-chain intermediate form. Further breakdown of the caffeine may lead to production of glyoxalic acid, monomethyl urea and dimethyl urea. The authors proposed this mechanism based on NMR, HPLC, and GC-MS analysis of the byproducts. They also measured the amount of oxygen consumed by the microorganisms in the presence of various substrates,

concluding that substrates causing oxygen consumption could be oxidized as part of this pathway.

In these experiments, *Klebsiella* and *Rhodococcus* were not separated, so there may be some sort of complimentary effect between bacteria necessary for the full oxidative pathway. Soil was used as the source and the bacteria were selected by growing them "using caffeine as the sole source of carbon," though no broth or agar recipes are reported. During experiments, cultures were grown with 0.05% g/L of caffeine and 0.1% glucose; the glucose was reportedly added to help speed up the reaction. It is unclear if glucose aided the reaction by giving the bacteria some initial substrate to encourage early growth or if the glucose was otherwise necessary for some other purpose in the oxidation reactions. The mixture of bacteria was able to oxidize 100% of the initial caffeine (present at 100 mg/mL) into 1, 3, 7-trimethyluric acid in 10 h (Madyastha and Sridhar 1998).

Biodecaffeination of Black Tea Dhool

Babu and others (2012a) explored the optimization of relevant parameters to create a biodecaffeination process that is 80% effective in black tea. The author's major hypothesis was that biodecaffeination could be applied to an actual food product on a less-than-industrial yet still relevant scale. At that time, studies had only looked at biodecaffeination of pure caffeine solutions, which are obviously much less complex than actual food systems. The authors furthermore identified several aspects of the procedure which they considered "bottlenecks" of this technology. The major parameters studied were: enzyme concentration, physical treatments, moisture content, temporal addition of

enzyme, mixing, oxygenation, caffeine-polyphenol interactions, and protein-polyphenol binding.

The methodology of the experiment begins through the creation of a decaffeinating cell-free extract. Previous research conducted by the same authors determined that caffeine demethylase enzymes could be stabilized in the cell-free extract from *Pseudomonas alcaligenes* MTCC 5264. Cells were inoculated in sucrose growth medium that was spiked with caffeine after 48 h to encourage demethylase activity. Cells were then harvested at 96 h, as it was found that they would enter a death phase after 108 h of growth. The presence of the demethylase was correlated with HPLC analysis of caffeine remaining in solution; it was assumed that less caffeine meant greater enzyme production. Biomass concentration was determined to be 0.64 mg/mL via spectrophotometric measurements. The cell-free extract was produced by centrifuging the cells, removing the supernatant, washing the resulting pellet with saline buffer, and inoculating with the following stabilizing agents: lysozyme, phenyl methyl sulfonyl fluoride, dithiothreitol, and glycerol. The first several studies were conducted using pure caffeine solutions to ensure that the extract was properly prepared.

From there, the cell-free extract was inoculated with black tea dhool, a fermenting intermediate produced during tea processing, under various conditions. Rates of decaffeination were determined by measuring caffeine concentration via HPLC analysis. Other methyl xanthine intermediates, such as theobromine and allantoin, were similarly measured as further evidence of caffeine degradation. Frequently the authors report this data as “% biodecaffeination” which was calculated as caffeine metabolized divided by

initial caffeine. Particular parameters were controlled or manipulated on an individual basis.

The first variable considered was the volume of the cell-free extract in order to optimize enzymatic activity, especially of the caffeine demethylase. This enzyme removes the methyl group from the C-1 carbon of caffeine to form theobromine, which itself is a stimulant naturally found in various foods. The reaction requires the oxidation of NADH to NAD^+. The cleaved methyl group is released into solution as formaldehyde. Caffeine demethylase is still not greatly understood, as it has yet to be fully sequenced or to have its three-dimensional structure determined (Gummadi and others 2012). Other potential pathways for caffeine degradation, such as oxidation or enzymatic metabolism to other products, were not reported in this research.

Enzymatic activity was found to plateau at a concentration of approximately 10000 U (enzyme unit, measured as the amount of enzyme needed to catalyze the conversion of μmol caffeine/min) per 100 g of dhool substrate. This maximum corresponded to 61.21% biodecaffeination in 90 min. This lower percentage of decaffeination, especially compared to the 80% figure later reported, is due to the lack of optimization of other parameters at this point in the study. The authors reasoned that lower enzymatic concentrations would not be sufficient to metabolize the high levels of caffeine in the dhool solution, and that moisture loss after 90 min would reduce demethylase activity. This hypothesis is based on a similar trend observed in polyphenol oxidase activity, during dhool fermentation. The maximum at 10000 U/100 g was assumed to be a result of the “non availability of caffeine to the enzyme.” This implies that almost all caffeine was being converted at all times at enzymatic concentrations

greater than 10000 U/100 g, and that the rate-limiting aspect of the reaction is no longer substrate availability.

When biodecaffeination was performed with empirically determined optimal parameters, the process reduced the total caffeine of the tea dhool by 80% (Figure 3). There was a clear maximum of activity in the first 15 min along with a slight increase at the 75 min mark, possibly owing to intermittent enzyme addition at 60 min. Enzymatic activity was also at its maximum around 15 min, reaching a capacity of 3000 U/mL. After 90 min, enzyme activity dropped to nearly 0 U/mL, thus halting biodecaffeination at around 80%. The authors point to the formation of caffeine-polyphenol and protein-polyphenol complexes as the major reasons for this plateau effect.

New Sources and Microbes

Microbial methods for decaffeination have the potential to be cheaper, more efficient, and more effective at removing caffeine from foods than traditional methods due to the potential of reusing enzymes and the avoidance of chemical solvents (Gummadi and others 2012). In addition, these methods could be used to process caffeine-rich waste that cannot be recycled into the environment. By continuing to determine the breadth of organisms that might have the potential to degrade caffeine, microbial decaffeination methods could be made more efficient and food-safe.

Even though there is a wealth of microorganisms that have been shown to break down caffeine, it has been argued that still “more microorganisms, which could degrade caffeine, need to be isolated” (Babu 2007), and we theorized that the use of compost as a new source will help to expand this technology.

Our objective was to isolate and identify microorganisms from compost and to investigate their potential as biodecaffeinators. Compost samples from various sources were used to isolate a caffeine-metabolizing organism on a selective agar that contained caffeine as its sole carbon source. We hypothesized that by using a variety of samples for microbial sources we could isolate other microorganisms that may be more efficient and safe for use in the biodecaffenation of foods.

Materials and Methods

Compost was either isolated from the compost bin at a local café (Libe Café, Ithaca, NY) or from compost processing facilities (Cornell Cooperative Extension, Ithaca, NY; Cayuga Compost, Trumansburg, NY). Compost produced without the addition of worms is hereafter referred to as garden compost; compost with added worms is referred to as worm compost. Subsamples of 1 g of compost were added to standard nutrient broth (VWR, Philadelphia, PA) and incubated at 35ºC for 2 d. Broth cultures were streaked onto caffeine agar plates (Baker and others 2012) that contained caffeine as the sole carbon source along with other minerals necessary for growth (Table 1) and incubated at 35ºC. Isolates were re-streaked onto pure culture caffeine agar plates through at least two purification steps to confirm caffeine fermentation.

The identity of resulting colonies was determined using 16S rDNA PCR amplification, gel electrophoresis, and sequencing analysis. A standard PCR kit was used for all reagents (Amplitaq Life Sciences, Grand Island, NY), with the exception of the 16s rDNA primers (Integrated DNA Technologies, Coralville, IA) (Table 2). Thermocycling time and temperature conditions were as follows: initial hold of 10 min at

95° C; 30 cycles of 1 min at 95°C, 1 min at 50°C, and 1.5 min at 72°C; cool down for 7 min at 72°C; and final hold at 4°C (Applied Biosystems 2720 77560 thermocycler). Resulting 16s rDNA sequences were matched against a BLAST database for microbial identification (>99% certainty).

Results and Discussion

The following bacteria, including their source location, were determined through 16S rDNA sequencing: *Arthrobacter arilaitensis* strain Re117 (from garden compost), *Paenibacillus cookii* strain LMG 18419 (from worm compost), *Pseudomonas fragi* strain ATCC 4973 (from garden compost), *Pseudomonas monteilii* strain CIP 104883 (from both garden and worm compost), *Pseudomonas plecoglossicida* strain FPC951 (from garden compost), *Pseudomonas putida* strain KT2440 (from both garden and worm compost), *Pseudomonas rhizospaerae* strain IH5 (from garden compost), *Sphingobacterium mizutaii* strain DSM 11724 (from garden compost), *Streptomyces carpaticus* strain NRRL B-16359 (from café compost), and *Streptomyces sampsonii* strain ATCC 25495 (from garden compost) (Table 3).

Arthrobacter are Gram-positive obligate aerobic bacteria commonly found in soil. One helpful function of *Arthrobacter* is their ability to degrade pesticides in the soil, which has made them useful in waste management technologies such as bioremediation. *Arthrobacter* are also known for their ability to use N-heterocyclic compounds as their sole carbon source for growth. For example, O'Loughlin and others (1999) found that *Arthrobacter* species were able to degrade 2-methylpyridine, 2-ethylpyridine, and 2-

297 hydroxypyridine, though ammonium was produced as a byproduct. This evidence is
298 consistent with our observation of *Arthrobacter*'s caffeine-metabolizing activity.
299 *Paenibacillus* are Gram-positive, rod-shaped, spore-forming, non-pathogenic
300 facultative anaerobic bacteria (Rieg and others 2010). Common sources of *Paenibacillus*
301 include the soil, water, rhizosphere of plant roots, and food (Scheldeman and others
302 2004). Due to the wide range of extracellular enzyme and secondary metabolite
303 production of the *Paenibacillus* genus, they may have industrial, agricultural, and
304 medical uses. For example, *P. polymyxa* was found to produce (2R,3R)-2,3-butanediol
305 dehydrogenase, which allows for the production of enantiopure (2R,3R)-2,3-butanediol
306 for pharmaceutical use (Yu and others 2011). Xylanase enzymes produced by various
307 *Paenibacillus* species may also have application in beverages and baking technologies
308 (Khianngam and others 2012). However, no previous research has looked at a link
309 between *Paenibacillus* and caffeine metabolism. *Paenibacillus* has been shown to
310 degrade agar (Hosoda and others 2003), which challenges this study's assumption of
311 caffeine metabolism, as the agar would be another viable carbon source for *Paenibacillus*
312 growth. Additional experiments would be needed to confirm caffeine metabolism.
313 *Sphingobacterium* are Gram-negative, non-sporulating rod-shaped bacteria named
314 after their unique sphingophospholipids (Yabuuchi and others 1983). Cotton-waste
315 compost has previously been documented as a source for *Sphingobacterium* (Yoo and
316 others 2007). However, some *Sphingobacterium* ssp. have been shown to be pathogenic
317 and can cause septicemia (Freney and others 1987), making them non-ideal bacteria for
318 food use.

Streptomyces are spore-forming, Gram-positive, facultative anaerobic bacteria commonly found in the soil (Morosoli and others 1997). The genome of *Streptomyces* species is large allowing for the production of various enzymes and the ability to degrade multiple different substrates. As a result, *Streptomyces* species are widely used in the production of antibiotics and extracellular proteins for both medicinal and agricultural applications. For example, *Streptomyces* has been investigated as a potential producer of xylanase enzymes, which have multiple applications in the food industry and in other biotechnologies (Morosoli and others 1997). Previous research has shown that *Streptomyces* species will grow and produce protein in the presence of high caffeine concentrations (Gummadi and others 2012). To our knowledge, this is the first time that *Streptomyces* has been documented to utilize caffeine as a carbon source and thus degrade it.

Gram-positive organisms and extracellular protein production

Biodecaffeination technologies, as applied to foods thus far, cannot use whole-cell fermentation, as there are many substrates in foods that would also be utilized by the microorganism, causing deleterious effects to the foods' quality. Therefore, all reports of biodecaffeination on food systems have described the isolation of a cell-free extract containing the caffeine demethylase enzymes and have used this extract for caffeine removal (Babu 2007; Babu and others 2012a). This requires that the cells be lysed and centrifuged (at cold temperatures, so as to not denature the heat labile enzymes) to separate the cellular components from the enzymatic extract. However, future

technologies might be designed to harvest the required enzymes from the extracellular space surrounding the bacteria.

It has been suggested that Gram-negative microorganisms, such as *Escherichia coli*, are less effective producers of extracellular proteins for industrial use than Gram-negative species (Binnie and others 2003; Pierce and others 2002; Stammen and others 2010). Gram-negative species produce lipopolysaccharides in their outer membranes that must be separated from any enzymatic extract that would come into contact with food (Stammen and others 2010). In this respect, the Gram-positive microorganisms *Arthrobacter*, *Paenibacillus*, and *Streptomyces* may prove useful in the expression of extracellular caffeine demethylase enzymes. *Streptomyces* has been specifically targeted as a potentially effective producer of extracellular enzymes, especially in comparison to Gram-negative species like *E. coli* (Binnie and others 2003). Pierce and others (2002) compared the specific production of α-amylase enzymes in *E. coli* and *Streptomyces lividans*. Though *S. lividans* had some disadvantages, such as a slower fermentation and a lessened production when in the presence of a complex substrate, it was overall found to be more favorable for use in protein production than *E. coli*. This finding was largely a result of a higher specific activity in the enzymatic extract produced by *S. lividans*. Enzymes produced by *E. coli* also required added post-processing to remove various cellular components, but even after post-processing the enzymes from *E. coli* were only 64% as effective. Other research has also shown that extracellular proteins produced by *Streptomyces* species may be easier to harvest in their properly folded, un-denatured states (Binnie and others 2003).

Conclusions and suggestions for further research

This project determined that *Arthrobacter*, *Paenibacillus*, and *Streptomyces* might be useful in biodecaffeination technologies and could be isolated and cultured from compost samples. *Sphingobacterium*, Gram-negative bacteria, were also determined to grow on caffeine-containing media. Further research should investigate the rate of caffeine degradation of these bacteria and determine optimal conditions (e.g. pH, temperature, agitation, aeration) at which decaffeination would occur, especially in relation to *Pseudomonas* and other previously determined decaffeinating microorganisms. The economic, environmental, health, and industrial sustainability of such a novel biotechnology would also need to be determined.

Several limitations inherent to the experimental design of this study allow for alternate explanations for the results, necessitating further studies before caffeine metabolism can be fully confirmed. For one, the compost samples were initially grown in an all-purpose nutrient-rich broth. This step was intended to encourage microbial growth among all of the species in solution, prior to streaking on the selective caffeine agar. However, it may have led to the caffeine-metabolizing microorganisms being outcompeted by other, more robust and rapidly growing species. A more effective procedure may have involved growing the samples in either nutrient broth spiked with some level of caffeine or caffeine liquid medium broth, which follows a similar recipe to the caffeine agar plates (Table 1) but does not contain agar.

Furthermore, the proper controls were not employed during screening of the microorganisms. Nutrient broth cultures were only streaked onto caffeine agar plates (Table 1) and were not streaked onto all-purpose nutrient agar or brain-heart infusion

type plates. This did not allow for the screening of all microorganisms present in the compost samples.

The assumption of caffeine as the only usable carbon source may also be unfounded, as several microorganisms have been previously found to degrade agar (Hosoda and others 2003). It is therefore possible that the microorganisms were instead using the agar as a carbon source for metabolic activities. *Paenibacillus* species have been found to degrade agar, challenging our proposal of *Paenibacillus cookii* as a potential microorganism for biodecaffeination (Hosoda and others 2003). A better control would have been to streak the colonies simultaneously on agar plates without caffeine that would otherwise contain the same ingredients as the caffeine agar plates, to ensure that the caffeine was necessary for the growth of the microorganisms.

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410 **References**

411 Babu VRS. 2007. A biotechnological approach for decaffeination. [PhD thesis]. Mysore,
412 India: University of Mysore. 323 p.
413 Babu VRS, Thakur MS, Patra S. 2012a. Effect of physicochemical parameters on
414 enzymatic biodecaffeination during tea fermentation. J Applied Biochemistry and
415 Biotechnology 166:112-26.
416 Babu VRS, Thakur MS, Patra S. 2012b. Structure prediction of caffeine demethylating
417 enzyme from *Pseudomonas alcaligenes*. Int Proceedings of Chemical, Biological,
418 and Environmental Engineering 29:59-63.
419 Baker S, Sahama S, Rakshith D, Kavitha HU, Kavitha KS, Satish S. 2012.
420 Biodecaffeination by endophytic *Pseudomonas* sp. isolated from *Coffee arabica*
421 L. J Pharmacy Research 5(7):3654-7.
422 Binnie C, Cossar JD, Stewart DIH. 1997. Heterologous biopharmaceutical protein
423 expression in *Streptomyces*. Trends in Biotechology 15:315-20.
424 Freney J, Hansen W, Ploton C, Meugnier H, Madier S, Bornstein N, Fleurette J. 1987.
425 Septicemia caused by *Sphingobacterium multivorum*. J Clinical Microbiology
426 25(6):1126-8.
427 Gummadi SN, Bhavya B, Ashok N. 2012. Physiology, biochemistry, and possible
428 applications of microbial caffeine degradation. Applied Microbiology and
429 Biotechnology 93:545-54.
430 Hosoda A, Sakai M, Kanazawa S. 2003. Isolation and characterization of agar-degrading
431 *Paenibacillus* spp. associated with the rhizosphere of spinach. Bioscience
432 Biotechnology and Biochemistry 67(5):1048-55.

433 Khianngam S, Akaracharanya A, Visessanguan W, Kim K, Lee KC, Lee J, Tanasupawat
434 S. 2012. Characterization of xylanolytic *Paenibacillus* strains isolated in
435 Thailand. Int J Bioassays 1(11):144-9.
436 Lee S, Park MK, Kim KH, Kim YS. 2007. Effect of supercritical carbon dioxide
437 decaffeination on volatile components of green teas. J Food Sci 72:S497-502.
438 Madyastha KM, Sridhar GR. 1998. A novel pathway for the metabolism of caffeine by a
439 mixed culture consortium. Biochemical and Biophysical Research
440 Communications 249:178-81.
441 Mazzafera P, Olsson O, Sandberg G. 1996. Degradation of caffeine and related
442 methylxanthines by *Serratia marcescens* isolated from soil under coffee
443 cultivation. Microbial ecology 31:199-207.
444 Morosoli R, Shareck F, Kluepfel D. 1997. Protein secretion in streptomycetes. FEMS
445 Microbiology Letters 146:167-74.
446 O'Loughlin EJ, Sims GK, Traina SJ. 1999. Biodegradation of 2-methyl, 2-ethyl, and 2-
447 hydroxypyridine by an *Arthrobacter* sp. isolated from surface sediment.
448 Biodegradation 10(2):93-104.
449 Pierce JJ, Robinson SC, Ward JM, Keshavarz-Moore E, Dunnill P. 2002. A comparison
450 of the process issues in expressing the same recombinant enzyme periplasmically
451 in *Escherichia coli* and extracellularly in *Streptomyces lividans*. J Biotechnology
452 92:205-15.
453 Rieg S, Bauer TM, Peyerl-Hoffmann G, Held J, Ritter W, Wagner D, Kern WV, Serr A.
454 2010. *Paenibacillus larvae* bacteremia in injection drug users. Emerging
455 Infectious Diseases 16(3):487-9.

456 Sauer M. 1982. Comparison of the cytochrome P450 containing monooxygenases
457 originating from two different yeasts. Developing Biochemistry 23:452-7.
458 Scheldeman P, Goossens K, Rodriguez-Diaz M, Pil A, Goris J, Herman L, De Vos P,
459 Logan NA, Heyndrickx M. 2004. *Paenibacillus lactis* sp. nov., isolated from raw
460 and heat-treated milk. Int J Systematic and Evolutionary Microbiology 54:885-91.
461 Schwimmer S, Kurtzman Jr. RH, Heftmann E. 1971. Caffeine metabolism by *Penicillium*
462 *roqueforti*. Archives of Biochemistry and Biophysics 147:109-13.
463 Stammen S, Müller BK, Korneli C, Biedendieck R, Gamer M, Franco-Lara E, Jahn D.
464 2010. High-yield intra- and extracellular protein production using *Bacillus*
465 *megaterium*. Applied and Environmental Microbiology 76:4037-46.
466 Woolfolk CA. 1975. Metabolism of *N*-methylpurines by a *Pseudomonas putida* strain
467 isolated by enrichment of caffeine as the sole source of carbon and nitrogen. J
468 Bacteriology 123:1088-106.
469 Yabuuchi E, Kaneko T, Yano I, Moss W, Miyoshi N. 1983. *Spingobacterium* gen. nov.,
470 *Sphingobacterium spiritivorum* comb. nov., *Sphingobacterium multivorum* comb.
471 nov., *Sphingobacterium mizutae* sp. nov., and *Flavobaterium indologenes* sp.
472 nov.: Glucose-nonfermenting Gram-negative rods in CDC groups IIK-2 and IIb.
473 Int J Systematic Bacteriology 33(3):580-98.
474 Yamaoka-Yano DM, Mazzafera P. 1999. Catabolism of caffeine and purification of a
475 xanthine oxidase responsible for methyluric acids production in *Pseudomonas*
476 *putida* L. Revista de Microbiologia 30:62–70.

Yoo S, Weon H, Jang H, Kim B, Kwon S, Go S, Stackebrandt E. 2007. *Sphingobacterium composti* sp. nov., isolated from cotton-waste composts. Int J Systematic and Evolutionary Microbiology 57:1590-3.

Yu B, Sun J, Bommareddy RR, Song L, Zeng A. 2011. Novel (2R,3R)-2,3-butanediol dehydrogenase from potential industrial strain *Paenibacillus polymyxa* ATCC 12321. Applied and Environmental Microbiology 77(12):4230-3.

Yu CL, Kale Y, Gopishetty S, Louie TM, Subramanian M. 2008. A novel caffeine dehydrogenase in *Pseudomonas* sp. strain CCB1 oxidizes caffeine to trimethyluric acid. J Bacteriology 190:772-6.

Figure 1: Proposed mechanism for the metabolism of caffeine by *Pseudomonas putida*, reproduced from Gummadi and others (2012).

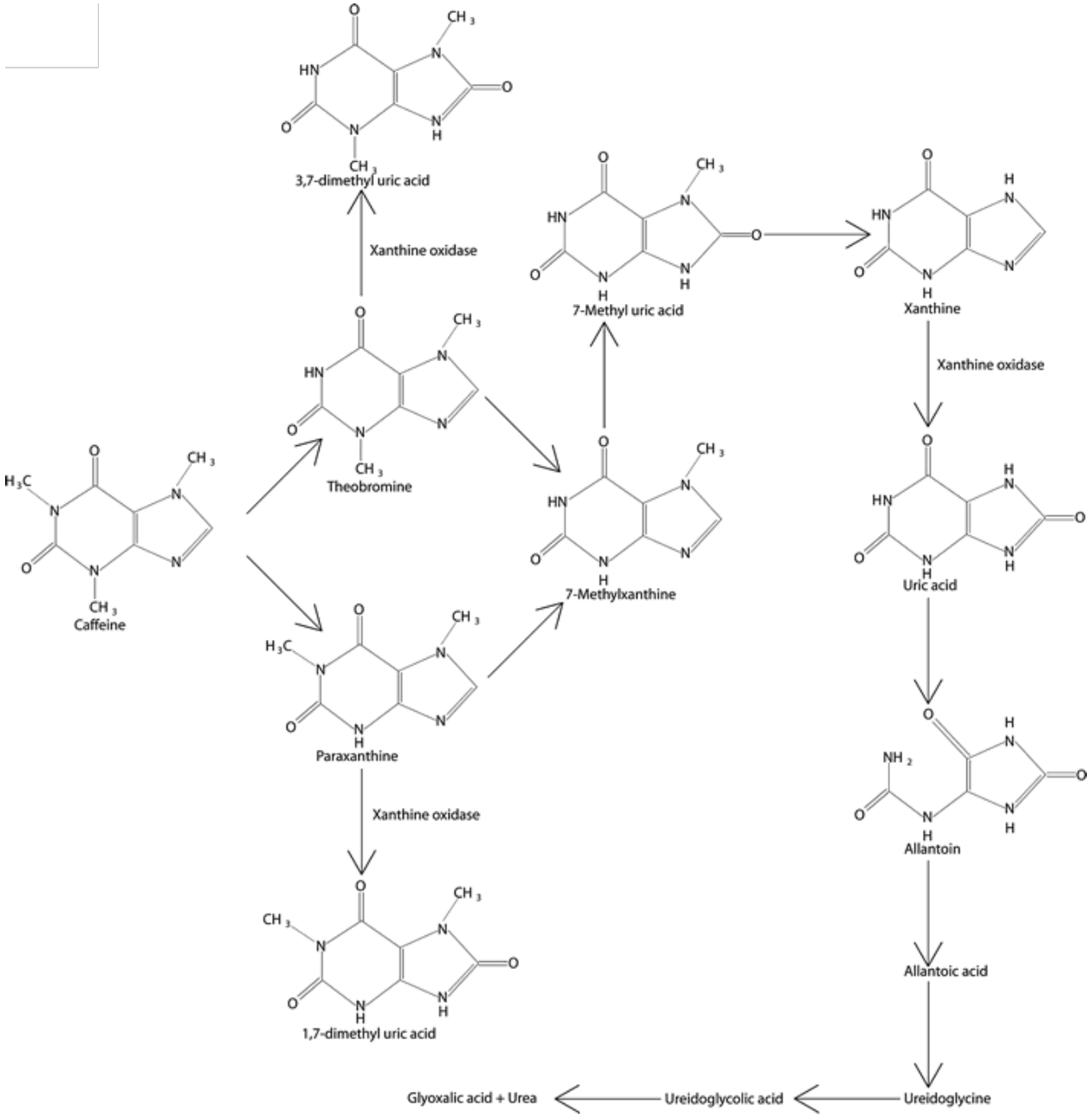
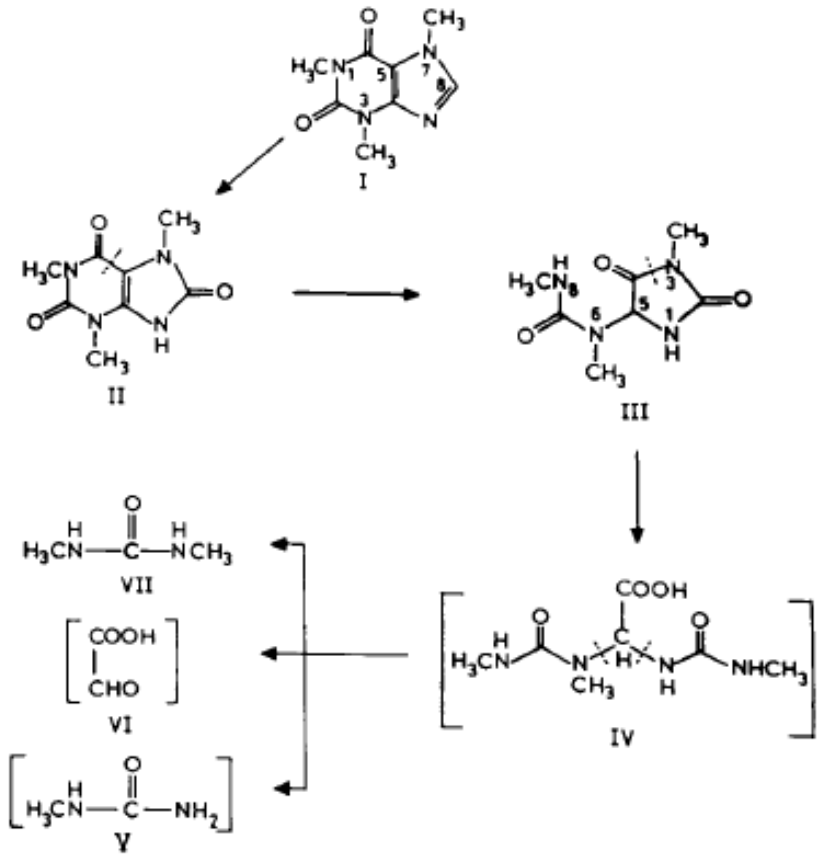


Figure 2: Proposed pathway for the oxidation of caffeine by a mixture of *Klebsiella* and *Rhodococus* bacteria.



519 **Figure 3**: Residual enzyme activity and percent biodecaffeination of black tea dhool over
520 time, reproduced from Babu and others (2012a).

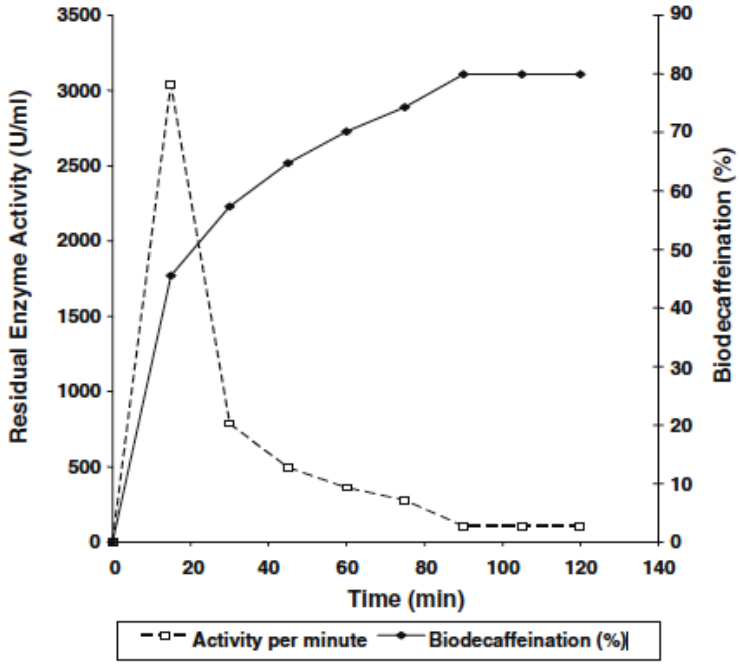


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Table 1: Concentration of ingredients in the caffeine agar (Baker and others 2012).

Ingredient	Concentration
$Na_2HPO_4 \cdot 7H_2O$ (Mallinckrodt, Paris, KY)	6.40 g/L
KH_2PO_4 (Sigma-Aldrich, St. Louis, MO)	1.50 g/L
NaCl (Fischer Scientific, Fair Lawn, NJ)	0.25 g/L
NH_4Cl (Fischer Scientific, Fair Lawn, NJ)	0.50 g/L
Agar (Difco, Sparks, MD)	15.0 g/L
Caffeine (Sigma-Aldrich, St. Louis, MO)	3.50 g/L

Table 2: 16S rDNA primer sequences for PCR analysis (Integrated DNA Technologies, Coralville, IA).

Primer Type	Genetic Code
PEU7	5'-GCAAACAGGATTAGATACCC-3'
P3SH	5'CTACGGTTACCTTGTTACGACTT-3'

568 **Table 3:** Source location and previous documentation for the isolated microorganisms.

Species	Compost Source	Previously identified
Arthrobacter arilaitensis	Garden	No
Paenibacillus cookii	Worm	No
Pseudomonas fragi	Garden	Yes[1]
Pseudomonas monteilii	Garden and worm	Yes[1]
Pseudomonas plecoglossicida	Garden	Yes[1]
Pseudomonas putida	Garden and worm	Yes[1]
Pseudomonas rhizosphaerae	Garden	Yes[1]
Sphingobacterium mizutaii	Garden	No
Streptomyces carpaticus	Café	No[2]
Streptomyces sampsonii	Garden	No[2]

569 [1](Gummadi and others 2012)

570 [2]*Streptomyces* was found to increase the protein content of coffee pulp if used in
571 fermentation (Gummadi and others 2012), but there has been no previous documentation
572 of a decaffeinating activity.